Effects of Nicotine on Embryological Neural Development

A Senior Honors Thesis

Presented in Fulfillment of the Requirements for Graduation with Distinction in Biology in the College of Biological Sciences of the Ohio State University

by

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Abstract:

Over 1 million women, approximately 12-22% of the U.S. population, smoke while pregnant (Centers for Disease Control). Nicotine, the primary toxin in cigarette smoke has been associated with altered neural structure and functioning in rats (Roy, 2002) and premature birth, low birth weight, cognitive defects, and behavioral problems in humans (DiFranza and Lew,1995; Sexton, 1990; Olds, 1994).

The actions of nicotine are mediated by neuronal nicotinic acetylcholine receptors (nAChRs) which then influence synaptic transmission in many part of the vertebrate nervous system. In this study, zebrafish are being developed as a model for determining the effects of prenatal exposure to nicotine on motor neuron development through examination of genes expressed in the motor neuron pathway. Zebrafish provide the ideal molecular model as embryos are transparent, small, and are accessible at early ages. Recently it has been shown that transient exposure of zebrafish embryos to nicotine delays the development of secondary motor neurons (Svoboda et al., 2002).The mechanism of this action is not known.

This study examines the effect of nicotine on the expression of the LIM homeodomain-containing transcription factor Islet-1 (Isl-1), which is important for motor neuron differentiation (Pfaff et al., 1997). Consequently, we hypothesize that nicotine may affect motor neuron development by altering Islet-1 gene expression within motor neurons. To view anatomical expression patterns, in situ hybridization was used to compare control and nicotine treated embryos of the same ages. Further characterization

of the effects of nicotine on gene expression may be carried out focusing on the gene sonic hedgehog (SHH) which is expressed even earlier within the motor neuron pathway. The application of these findings may be used to solidify the link between smoking and motor neuron defects and diseases

Introduction:

A drug of abuse, nicotine is purported to have many effects on the vertebrate nervous system. Though correlated with many negative health effects, approximately 12-22% of the population, or 1 million women, in the United States smoke while pregnant (CDC). Prenatal exposure to nicotine has been correlated with a number of abnormalities including spontaneous abortions, Sudden Infant Death Syndrome (SIDS), and low birth weight (DiFranza and Lew,1995), as well as behavioral, cognitive and intellectual impairment (Sexton, 1990; Olds, 1994).

The actions of nicotine are mediated by neuronal nicotinic acetylcholine receptors (nAChRs) which then influence synaptic transmission in many part of the vertebrate nervous system. The presence of many of these receptors at the embryonic stage indicates that they have some role in development. Nicotine has been implicated in retracting neurite outgrowth in chick ciliary ganglions (Pugh and Berg, 1994). In the developing chick nervous system, nAChR blocking agents are involved in the regulation of motoneuron survival and the extent to which they branch within the body and form synapses (Hory-Lee and Frank, 1995; Oppenheim et al., 2000). Additionally, it has been recently shown that prenatal exposure of embryonic zebrafish to nicotine delays the development of secondary motor neurons, leading to functional difficulties (Svoboda et al., 2002).These studies strongly suggest that nicotine, as mediated by nAChRs, has an effect on motor neuron development and outgrowth within the embryonic nervous system.

To this end, we have hypothesized that nicotine affects motor neuron development by altering the expression of Isl-1, a LIM homeodomain transcription factor responsible for differentiation of subsets of neurons, specifically distinguishing somatic and visceral motor neurons. As there is no known mechanism for how nicotine might affect motor neurons, it is possible that Islet-1, whose expression is upstream of other LIM homeobox genes in development of motor neurons, is the key component of the differentiation pathway affected.

In order to ascertain the effect of nicotine on the expression of Islet-1 in an embryonic nervous system, we used the zebrafish model. Zebrafish embryos are transparent for easy visualization, available in large quantities with little variation between each embryo, and have a short developmental period for long-term studies. The zebrafish genome is remarkably similar to the rat genome, and embryos are available at times in utero when rat embryos are inaccessible. Additionally, observing the effects of nicotine on the traditional rat embryo model is complicated by hypoxia effects caused by constriction of fetal arteries and veins by nicotine, whereas zebrafish embryos, which develop outside the uterus, are exempt from this complication.

Methods:

In this experiment, we chose to examine the effects of nicotine on Isl-1 expression of 48, 72, and 96 hours post fertilization (hpf) embryos using *in situ* hybridization for anatomical visualization.

Collection:

Wild-type AB* zebrafish embryos were collected and allowed to develop at 28.5 C and staged according to known methods (Kimmel et al., 1995). To facilitate visual examination during *in situ* hybridization,0.2mM phenylthiourea (PTU) was added to fishwater to inhibit pigment formation. For nicotine exposures, nicotine (50 μm of hydrogen tartrate salt solution) was added directly to PTU-containing embryo medium immediately after collection. This concentration represents approximately 15μm of nicotine free base. 30 zebrafish embryos of each exposure (control and nicotine) were harvested at 48, 72, and 96 hpf for *in situ* hybridization.

RNA Isolation and Riboprobe Synthesis

Zebrafish RNA was isolated from whole embryos using Trizol (Life Technologies) and reverse-transcribed using random primers with the Superscript III Preamplification System (Invitrogen). Islet cDNA was amplified using degenerate PCR primers targeted for specific sequence information. Minus DNA and minus RT reactions were also used to eliminate contamination concerns. The PCR products of 96hpf control and nicotine embryos were then examined via gel electrophoresis.

Zebrafish Islet-1 cDNA from 48, 72, and 96 hpf embryos (control and nicotine) was inserted into a PCR II TOPO vector (Invitrogen) and then transformed into Top 10 Chemically Competent E.coli Cells. Colonies were grown on ampicillin-containing agar plates. DNA was purified (Eppendorf Miniprep FastPlasmid systems) and linearized with PstI. DNA was precipitated by phenol-chloroform ethanol extraction. Antisense digoxigenin-labeled RNA probe was synthesized using SP6 polymerase (Roche DIG RNA labeling kit SP6/T7). This probe did not work during *in situ* hybridization procedure. It was digested using alkaline hydrolysis to remove secondary structures, yet still did not produce a result. The synthesis procedure was repeated using Islet-1 cDNA provided by Dr. Christine Beattie's lab with the following changes: linearization took place using EcoRI and the RNA probe was synthesized using T7 polymerase (Roche DIG RNA labeling kit SP6/T7).

In situ Hybridization

In situ hybridization was performed following the procedures outlined in Beattie et al. (1997), Sagerstrom et al. (1996), and Thisse et al. (1993) with some modifications. Briefly, 30 whole embryos were placed in 1.5 mL eppendorf tubes, and fixed in 8% paraformaldehyde/2X phosphate buffered saline (PBS) overnight, taken through graded methanol washes, treated with Proteinase K, post-fixed in paraformaldehyde, and hybridized with 200 ng RNA probe overnight at 68 C. After a series of sodium chloride citrate (SSC) washes, zebrafish embryos were incubated with a preabsorbed alkaline phosphatase-conjugated anti-digoxigenin secondary antibody overnight, and then washed with multiple phosphate buffered saline with tween 20% (PBT) rinses. Detection was

performed with nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrates for the alkaline phosphatase and the reaction was halted with PBS-Tween. Whole embryos were mounted using glycerol and viewed with a Zeiss Axioskop Widefield LM. Anatomical labeling was performed following outlines in Higashijima et al. (2000).

Results:

Upon visual examination of Isl-1 expression in total RNA of the embryo via polymerase chain reaction (PCR), there appeared to be no change in RNA expression of control and nicotine-treated 96hpf embryos (see Figure 1). In response to this, *in situ* hybridization was carried out to view anatomical Isl-1 expression. In 48hpf embryos a slight reduction in expression of Isl-1 was noted in the vagus nerve (CN X) and the trigeminal nerve (V) (see Figure 2). A reduction in Isl-1 expression was also seen in the trigeminal nerve (V), oculomotor nerve (CN III), facial nerve (CN VII), and most notably, the vagus nerve (CNX) in 72 hpf embryos (see Figure 3). A similar reduction of Isl-1 expression was noted in 96 hpf embryos in the trigeminal (CN V) and vagus nerve (CNX) (see Figure 4).

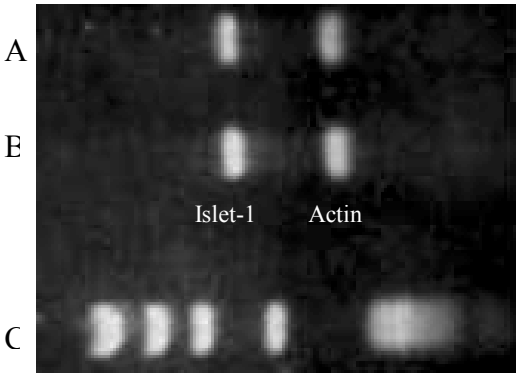


Figure 1. Zebrafish Islet-1 RNA Expression Detected Using PCR in 96hpf embryos

A. Nicotine-treated
B. Control
C. Molecular Marker ΦX

Figure 2. Zebrafish Islet-1 RNA Expression Detected Using *In Situ* Hybridization in 48h embryos.

A. Dorsal view with eyes visible laterally, 10X magnification, control v. nicotine treated

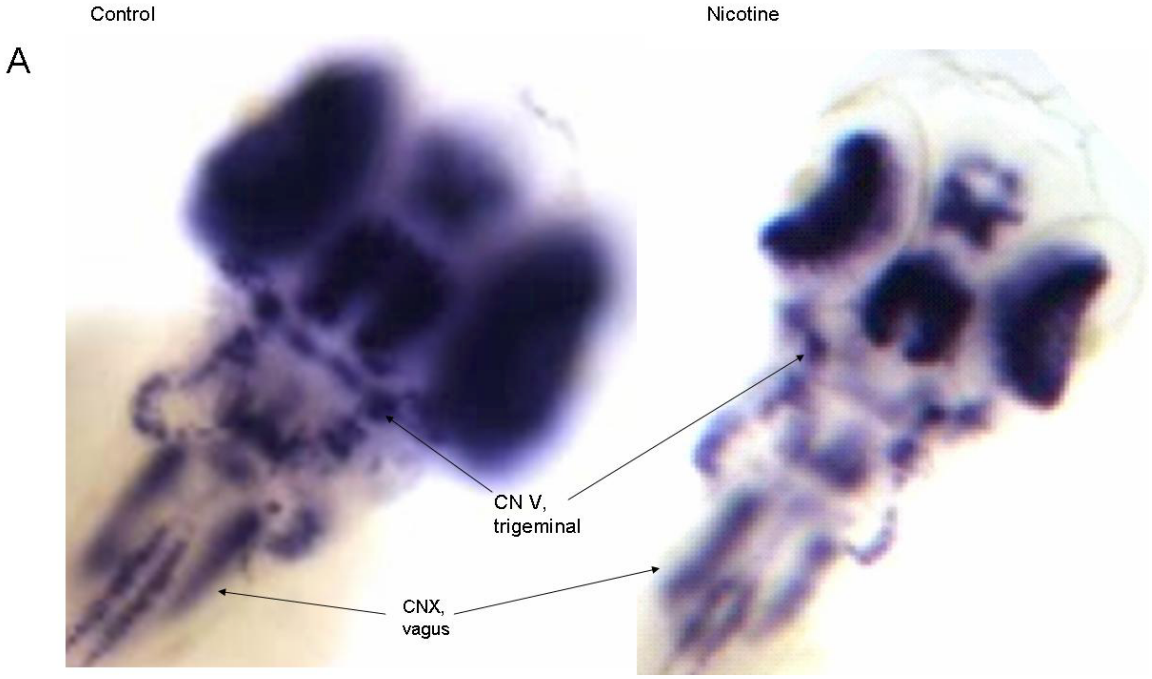
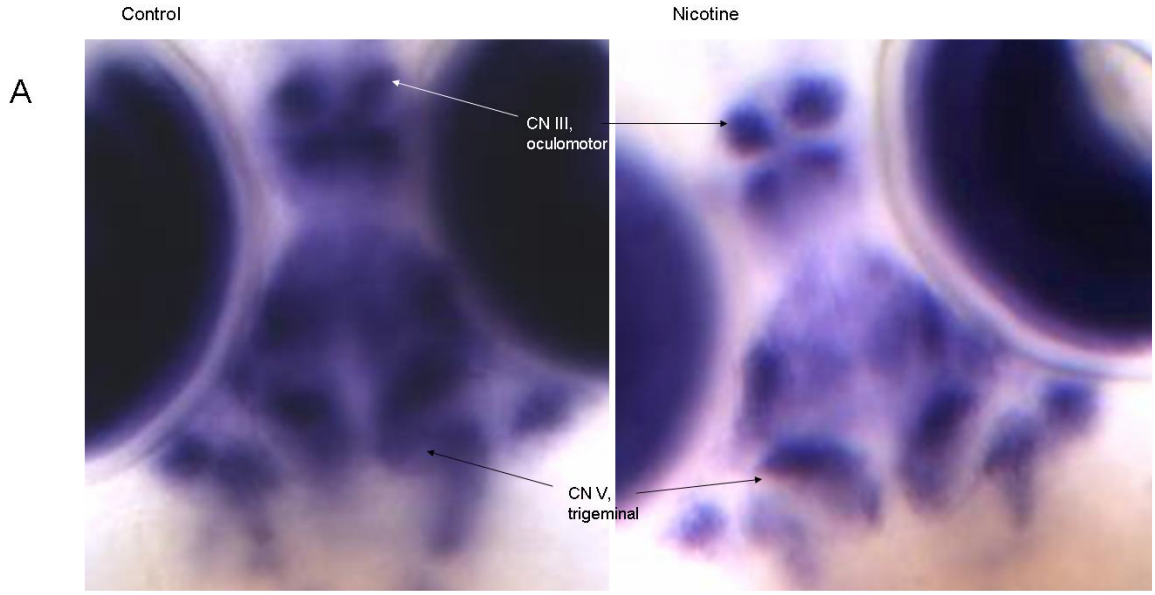
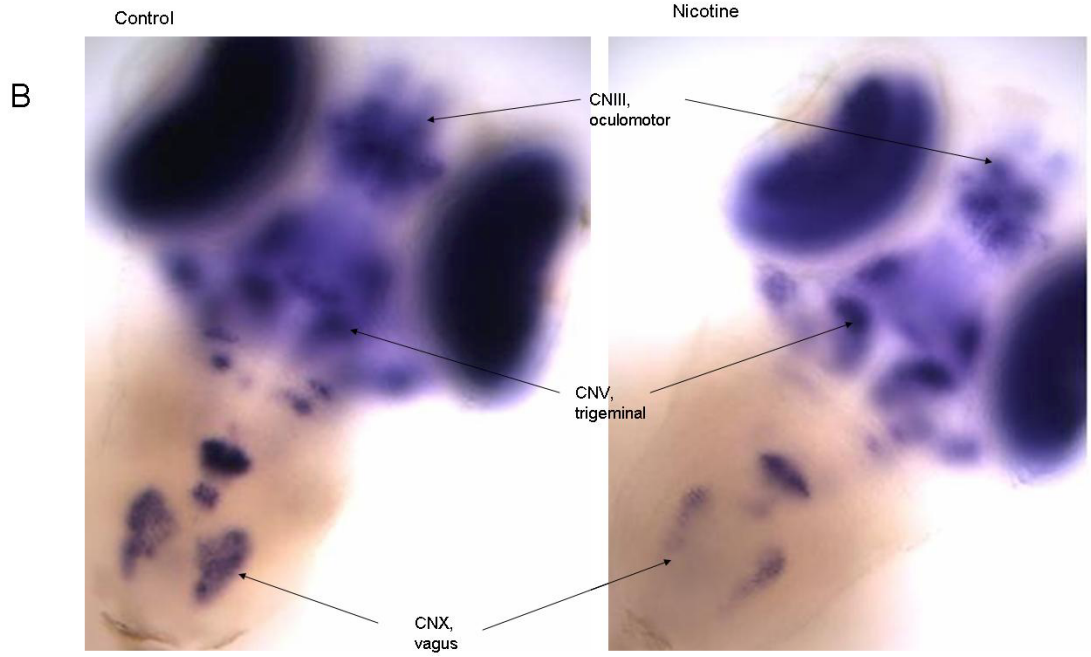


Figure 3. Zebrafish Islet-1 RNA Expression Detected Using *In Situ* Hybridization in 72h embryos.

A. Dorsal view with eyes visible laterally, 20X magnification, control v. nicotine treated
B. Dorsal view with eyes visible laterally, 10X magnification, control v. nicotine treated
C. Lateral view, 20X magnification, control v. nicotine treated





C

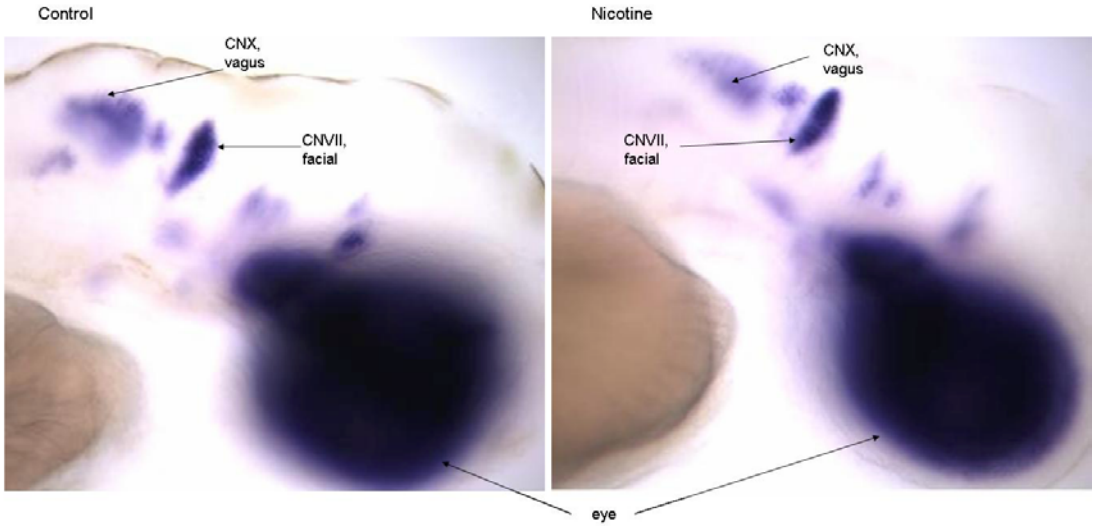
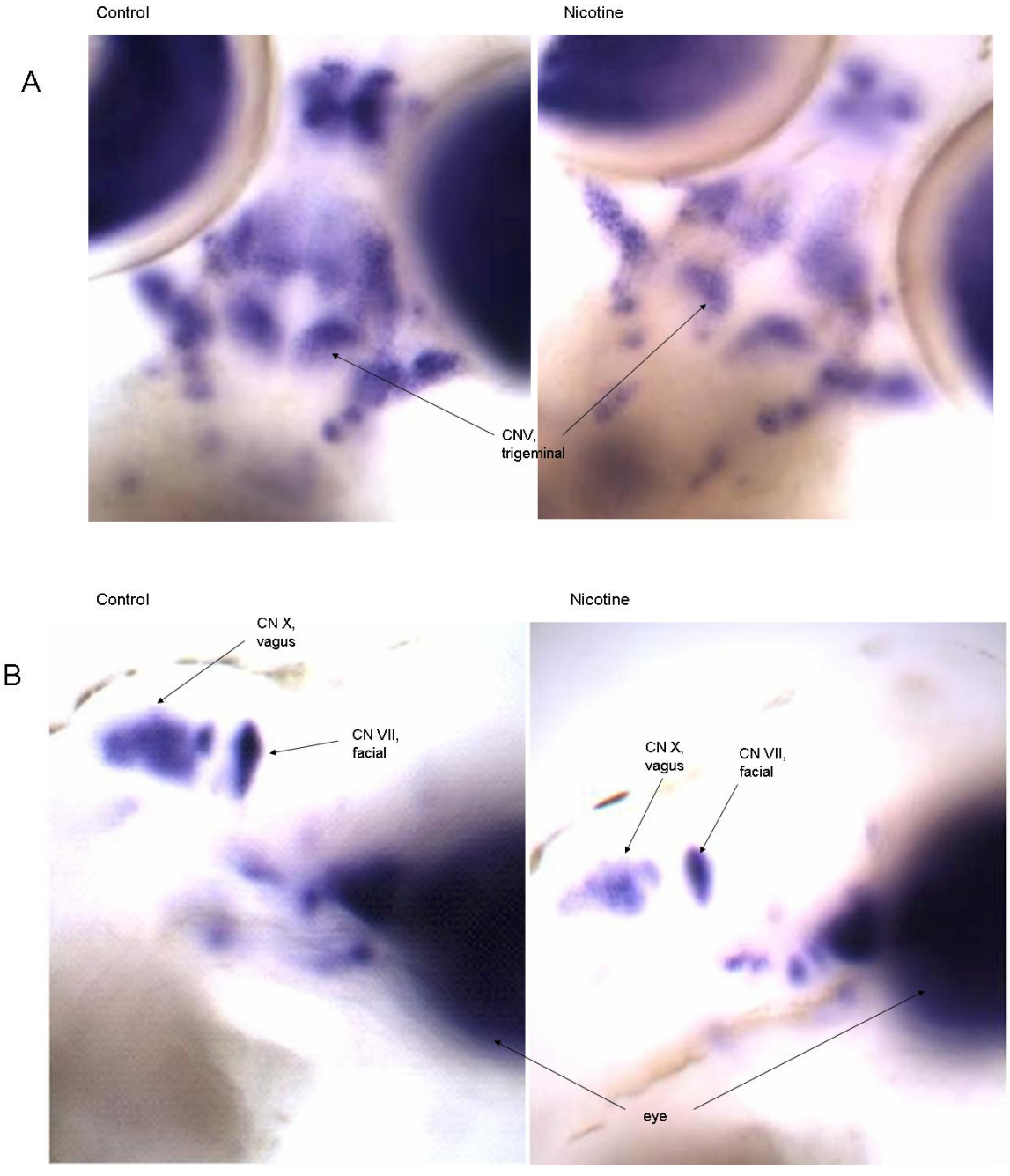

Control
Nicotine
CNX,
vagus
CNVII,
facial
CNX,
vagus
CNVII,
facial
eye

Figure 4. Zebrafish Islet-1 RNA Expression Detected Using *In Situ* Hybridization in 96h embryos.

A. Dorsal view with eyes visible laterally, 20X magnification, control v. nicotine treated
B. Lateral view, 20X magnification, control v. nicotine treated



Discussion:

There was no visible difference in expression of Isl-1 in control and nicotine treated embryos using RT-PCR for the entire embryo. Isl-1 expressing cells constitute a small population of total RNA for an embryo, so the Isl-1 signal may be masked by other cell populations when viewed using PCR. *In situ* hybridization permits visualization of a specific cell population in a whole embryo without this masking effect, as seen in Figures 2-4 above. Isl-1 expression was slightly reduced in specific cranial nerves, as seen through *in situ* hybridization, most noticeably the vagus nerve in nicotine-treated embryos. Nicotinic acetylcholine receptors have been noted to act on the vagus nerve to modulate immune response and inflammation (Ulloa, 2005), and the presence of these at the embryological level indicate a mechanism by which nicotine may act. Recently it has been shown that prenatal exposure to nicotine alters the types of embryological nAChRs involved in excitatory transmission within the vagal nerve (Huang 2004). Additionally, neonatal exposure to nicotine has been shown to desensitize nAChRs which modulate glutamergic transmission to hypoglossal motoneurons (Quitadamo 2005). The presence of these receptors at the embryological level as well as the receptiveness to nicotine exposure indicates a significant enough number of receptors may be available to interact with nicotine to modulate motorneuron growth and survival by affecting Isl-1 expression.

By decreasing Isl-1 expression, nicotine may affect the number of neurons that can differentiate into primary and secondary neurons. Though little is known about Islet function within the developmental pathway, Isl-1 expressing neurons may provide a path for other neurons to follow into differentiation. In the absence of this signal, significant

amount of neurons may be missing, which may contribute to neuromuscular defects and diseases.

In the larger sense, it is not known whether there is reduction of Isl-1 expression or a reduction in the number of Isl-1 expressing cells. Future studies may control for this by targeting a specific marker for this cell population. Additionally, it is not known whether nicotine is specifically affecting Isl-1 expression, or a transcription factor released upstream or downstream within the differentiation pathway. To this end, future studies may be directed at the Gli family of transcription factors, or sonic hedgehog, which mediates differentiation into motor or sensory neurons earlier in the developmental pathway (Vanderlaan, 2005).

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